REMARKS

35 U.S.C. 112

Claims 24, 26, 27, 29 and 30 are rejected under 35 U.S.C. Section 112 first paragraph as containing subject matter which was not described in the specification so as to enable one skilled in the art to make and or use the invention. Applicant notes that Examiner acknowledges the use of CD40L to inhibit secondary alloimmune response in an SCID experimental model thereby accepting the present data for the inhibition of platelet HLA alloimmune responses. Nevertheless Applicant submits that the present invention is not limited to platelet HLA alloimmune responses but to inhibiting alloimmune responses and in particular, HLA alloimmune responses.

The Examiner contends that the subject matter of the present claims is not enabled or supported by the described specification and contends that "*in vitro and animal model studies have not correlated well with in vivo clinical trial results in patients*". Correlations between in vivo animal models and in vivo clinical trials are difficult; nevertheless, the teachings of the present invention and the results data provided herein are based on an in vivo **humanized** animal model that is an accepted model for the correlation of in vivo animal studies to in vivo results in patients.

In this respect, Applicant submits a Declaration by the Inventor which confirms that the presently used humanized in vivo animal model is an accepted model in the present art; wherein the present humanized in vivo animal model is acceptable for the evaluation and correlation of immune responses in patients.

Applicant additionally submits several journal reference which further cite the humanized in vivo animal model of the present invention as an in vivo animal model which correlates the inhibition of a secondary human alloimmune response (*Transfusion*, **39,** Aug. 1999) (copy enclosed). The model of the present invention, as cited in Transfusion, was

additionally highlighted on the cover of the noted journal, further substantiating the present model as an accepted in vivo animal model for examining alloimmune responses in patients. Additional support for the use of the humanized in vivo animal model of the present invention for correlation to in vivo clinical trials is further supported by the use of said model by Semple JW, et al., *Blood* 2002, August 1;100(3): 1055-9 (copy enclosed).

Moreover, it is additionally noted in Bankert et al (*TRENDS in Immunology*, 2001), (copy enclosed), that the SCID mouse model is relevant to the evaluation of human therapies. More specifically, on page 389 (bottom paragraph on left) Bankert et al note that "*The SCID mouse models (particularly Models One to Three) have been used effectively to evaluate novel chemotherapeutic drugs and different drug-delivery systems. The results of these studies suggest that the data obtained from SCID mouse models correlate with observations in cancer patients; thus, the SCID mouse model might be useful for in vivo testing of new agents and methods of drug delivery...*". We note that the model of the present invention correlates to Model Two in Bankert et al. It is addionally noted on page 388 (bottom paragraph on left) that the model of the present invention is "*a model that is more clinically relevant*" thereby further substantiating the use of the present humanized in vivo animal model for the evaluation and correlation of alloimmune responses in patients.

Furthermore, Applicant refers the Examiner to the description pages that provide support for the use of the present humanized in vivo animal model for the correlation of said results to in vivo human results. The present description does support the subject matter of the claims, wherein CD40L is shown to inhibit a human alloimmune response and that the humanized mice were indeed producing human alloantibodies. More specifically, on page 9, line 30 it is noted that "*Group 3 made enhanced levels of alloantibody...*"; on page 10, line 2 "*CD40L active component was able to significantly decrease the human alloimmune antibody response to challenge with HLA mismatched blood cells in a humanized SCID model system*". Applicant

also refers the Examiner to page 14, line 10 which notes "*Antibody specificity for HLA antigens was confirmed using neat sera in the standard two-stage complement-dependent microlymphocytotocity assay using a typed panel of lymphocytes from donors...*" as further summarized in Table 1 on page 19 where antibodies in the humanized mouse model react with human lymphocytes, on page 16, line 14 it is further noted that "*...challenged mice made increasing levels of both allo-reactive IgG as well as IgM*". Furthermore, page 17, line 26 notes that "*The specificities of the alloantibodies produced...were determined by LCT and the results are shown in Table I*". In addition, on page 22, line 29 it is noted that "*the response of the mice consisted of alloantibodies to several HLA antigens...*". Applicant submits that the presently noted description pages do in fact provide enabling support for the use of CD40L to inhibit human alloimmune responses and that the humanized SCID mouse model of the present invention is an accepted model for the correlation to in vivo results in patients.

Moreover, the humanized animal model of the present invention does allow for the correlation to human in vivo results, as supported by the description on page 5, line 19 where it is noted that "*...there is provided an immunodeficient mouse model of human alloimmunization for testing in vivo effects of an immunotherapy or an inhibition of a human antibody response*". On page 21, line 28 the description notes "*An in vivo model of the secondary immune response was established in a Hu-PBL-SCID mouse model system using lymphocytes from previously sensitized individuals.*"

Applicant submits that the animal model of the present invention allows for such correlations since the present SCID animal model of the present invention is not simply an in vivo animal model, but is a humanized in vivo animal model, wherein a human immune system has been provided to an immunodeficient animal so as to thereby elicit human immune responses. Therefore the alloimmune responses established in the in vivo humanized animal model of the present invention allows for correlations to patients.

Accordingly, in connection with the above noted comments, the references cited and the Declaration submitted herewith, Applicant respectfully requests reconsideration and withdrawal of the present rejections.

Claim Objection

Applicant has amended claims 27 and 30 to overcome the informality objections noted therein.

35 U.S.C. 102(e)

Claim 27 is rejected under 35 U.S.C. 102(e) as being anticipated by Armitage et al. and Aruffo et al. Claim 27 has been amended, and claim 29 has been cancelled. Applicant respectfully requests reconsideration and withdrawal of the present rejection.

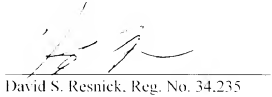
In view of the above and foregoing, it is respectfully submitted that the claims now on file are believed to be in condition for allowance, and prompt and favorable action is earnestly solicited. Should there be any question concerning this response or the application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of this application may be expedited.

Authorization is hereby given to the Commissioner to charge any defiant fees or to credit an overpayment to account number 50-0850.

Customer No. 26770

Respectfully submitted,

CANADIAN BLOOD SERVICES



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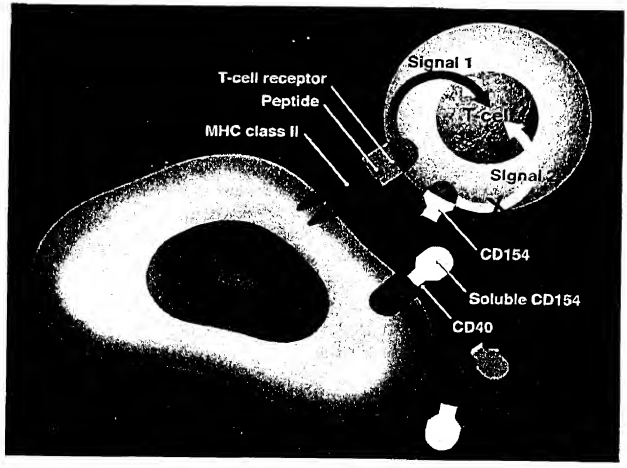
Encls. Declaration by the Inventor
1999 *Transfusion Core Page*
1999 Lazarus et al.
2002 Semple et al.
2001 Bankert et al..

Vol. 39, No. 8, August 1999

The Journal of the American Association of Blood Banks

TRANSFUSION



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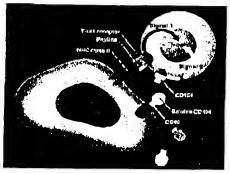

On the Cover:
Diagram depicting the potential mechanism of action by which soluble CD154 (CD40L) inhibits alloimmunization. Platelet alloimmunization is a T-cell-dependent immune response and requires T-cell activation via delivery of an activating signal from the T-cell receptor (Signal 1) in concert with a signal through a co-stimulatory molecule such as CD154 (Signal 2). Administration of a soluble form of CD154 inhibited the ability of CD40 on the antigen-presenting cell (APC) to engage CD154 on the T cell and inhibit T-cell activation and alloimmunization. Figure provided by Alan H. Lazarus, PhD, Department of Immunohematology, St. Michael's Hospital, Toronto, ON, Canada.

Inhibition of a secondary human alloimmune response via the soluble active component of CD154 (CD40L) in severe combined immune-deficient mice engrafted with human lymphocytes

A.H. Lazarus, A.R Crow, J. Freedman, V. Blanchette, and B. Hannach

BACKGROUND: Alloimmunization requires a process known as co-stimulation. An important co-stimulatory pathway for most immune responses is mediated by the interaction of CD40 on antigen-presenting cells with CD154 (CD40L) on host T cells. Blockade of this co-stimulatory pathway simultaneous with exposure to challenge with HLA-incompatible cells is hypothesized to inhibit alloimmunization.

STUDY DESIGN AND METHODS: Severe combined immune-deficient (SCID) mice were reconstituted with human peripheral blood lymphocytes (Hu-PBL-SCID mice) from a subject primed to HLA antigens and challenged with HLA-incompatible lymphocytes. Mice were challenged in the presence or absence of an 18-kDa soluble recombinant active form of human CD154 (18-kDa CD154). Human IgG production, alloimmunization, and in vitro T-cell responsiveness were assessed.

RESULTS: There was no significant effect of 18-kDa CD154 on human IgG levels in these mice, but it inhibited the development of HLA-specific alloantibody in this model to five subsequent untreated white cell challenges. In vitro T-cell proliferation in a mixed lymphocyte culture was also prevented by 18-kDa CD154.

CONCLUSION: The recombinant protein 18-kDa CD154 inhibited the ability of the Hu-PBL-SCID mice to mount a secondary immune response to allostimulation. This implies that transfusion-induced alloimmunization utilizes CD40-CD154 co-stimulation and that blockade of this pathway can inhibit T-cell function and interfere with the development of alloimmunization.

The alloimmune response to HLA antigens is a major problem in clinical practice. Alloimmunization occurs when a recipient receives an HLA-incompatible platelet transfusion or an HLA-incompatible tissue transplant. In the case of alloimmunization to platelet transfusions, HLA antibodies are made in response to the administration of HLA-incompatible platelet concentrates. These platelet concentrates consist of platelets as well as contaminating white cells, which are thought to act as antigen-presenting cells (APCs) and to induce, or greatly augment, the HLA antibody response. Attempts to decrease platelet concentrate alloimmuniza-tion have focused on reducing the numbers of contaminating white cells in these concentrates or by inactivating the white cells by ultraviolet irradiation. Although these methods have proven to be at least partially

ABBREVIATIONS: APC(s) = antigen-presenting cell(s); Hu-PBL-SCID mice = human-peripheral blood lymphocyte-severe combined immune-deficient mice; 18-kDa CD154 = an 18-kDa soluble, recombinant, active, extracellular form (amino acids Glu-108 to Leu 261) of the human CD154 molecule, PBLs = peripheral blood lymphocytes; SCID mice = severe combined immune-deficient mice; Th_2 cells = helper T cells type 2.

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TRANSFUSION 1999;39:818-823.

successful, many patients continue to become alloimmunized, especially those who have already been primed to HLA antigens by pregnancy or transfusions.[1] We recently described an in vivo humanized mouse model for examining human HLA antibody responses in alloimmunization,[2] and we used this model to study the inhibition of alloimmunization via the inhibition of immune costimulation.

It is known that interference with immune co-stimulation can inhibit both cell-mediated and antibody mediated responses. In particular, the CD40-CD154 co-stimulatory system is necessary for immune responsiveness.[3-6] CD40 is found on a variety of APCs, including B lymphocytes, and CD154, which is the co-stimulatory ligand for CD40, is found on helper T-lymphocytes. Interactions between B-cell CD40 and helper T-cell CD154 lead to B-cell co-stimulation that greatly augments B-cell activation and antibody production.[7-11] CD40 can also deliver a negative signal to the B cell.[7,8] CD40-mediated activation signals have been induced in B cells via antibodies to CD40, soluble CD154 fusion proteins, and cells transfected with CD154 constructs.[5,7-11] The trimolecular CD154 fragment (18-kDa CD154) used in the current work has been shown to promote B-cell proliferation and differentiation, to rescue B cells from apoptosis, and to activate resting B cells in vitro, as assessed by CD25 and CD23 expression.[12-14] The administration of antibodies directed to CD154 in vivo has been shown to block murine thyroiditis,[15] lupus-like nephritis,[16] autoimmune hemolytic anemia,[17] T-cell-mediated alloreactivity,[18] and helper T-cell type 2 (Th_2) responses.[19]

We employed the previously described Hu-PBL-SCID mouse (severe combined immune-deficient [SCID] mice that received human peripheral blood lymphocytes [PBLs] from donors immunized to HLA antigens) model system to study the effects of in vivo injection of a recombinant human soluble component of CD154 (18-kDa CD154) to block alloimmunization via interference with immune co-stimulation. Alloimmunization was inhibited. The use of 18-kDa CD154 to inhibit alloimmunization has implications for blood transfusion immunology as well as organ transplantation-associated graft-versus-host disease.

MATERIALS AND METHODS

SCID mice

C.B-17 SCID virgin female mice (6-8 weeks of age) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed under gnotobiotic conditions in the St. Michael's Hospital research vivarium. Blood from the tail vein (300 µL) was collected into untreated microvette tubes (Sarstedt, Montreal, PQ, Canada), and the serum was separated after incubation for 2 hours at 22°C. Serum levels of endogenous murine IgG were determined by enzyme-linked immunosorbent assay, and animals with a serum murine immunoglobulin concentration exceeding 10 µg per mL were excluded from the study.

Reconstitution

Human PBLs were obtained from a female blood donor with a history of pregnancy and low levels of circulating HLA class I alloantibodies. She was blood group O, HLA A1, A3, B7, and B37 and had low stable levels of circulating HLA-A2 and HLA-B5 alloantibodies. All SCID mice were injected with 20 µL of anti-asialo GM_1 (Wako Pure Chemical Industries, Dallas, TX) 1 day before reconstitution and exposed to 200 cGy of γ radiation just before reconstitution to enhance cellular engraftment, as described previously.[2] Human PBLs (1×10^7/mouse) were isolated from whole blood by Percoll density centrifugation and injected into the peritoneal cavity of SCID mice essentially as described.[2]

SCID mouse challenge

Challenge PBLs from HLA-A2-positive humans were isolated from whole blood by Percoll density centrifugation as described,[2] and reconstituted SCID mice were challenged with 2500 cGy γ-radiated of the human PBLs. The first challenge consisted of 2×10^7 PBLs per mouse, and subsequent challenges were with 10^7 PBLs per mouse twice weekly for 3 weeks starting on the day of reconstitution. Hu-PBL-SCID mice that received 18-kDa CD154 were injected with 200 µg of the 18-kDa CD154 via the intraperitoneal route on the day of reconstitution only.

Antibody detection

Human and mouse IgG was detected by a standard ELISA, and alloantibodies were detected by flow cytometry, both as described previously.[2] Briefly, SCID mouse sera were diluted 1-in-10 and incubated with 2×10^5 fresh HLA-typed PBLs in a volume of 20 µL for 1 hour at 22°C. The cells were then washed twice and incubated at 22°C for 1 hour in the dark in 100 µL each of 1 µg per mL of affinity-purified fluorescein isothiocyanate-labeled $F(ab')_2$ Fcγ-specific antihuman IgG (Tago Biosource, Camarillo, CA). The cells were then washed twice and fixed in 1-percent paraformaldehyde in phosphate-buffered saline. Ten thousand cells were analyzed with a flow cytometer (FACSort, Becton Dickinson, San Jose, CA) employing an argon ion laser operating at 15-mW power and using software (Lysis II, Becton Dickinson). Background staining was assessed by comparison with serum obtained from each SCID mouse before any manipulation. Antibody specificity for HLA antigens was confirmed in the standard two-stage, complement-dependent microlymphocytotoxicity assay using a 30-lymphocyte typed panel; a positive result was defined as >20 percent lysis of target cells.

In vitro lymphocyte cultures

PBLs from the donor used to reconstitute the Hu-PBL-SCID mice were cultured in vitro (2×10^5/well) with the same γ-radiated PBLs used to challenge the mice (4×10^5/well), with or without 18-kDa CD154 for 72 hours in a final vol-

ume of 200 µl, in complete medium (RPMI-1640, University of Toronto Media Services, Toronto, ON, Canada) containing 10-percent fetal calf serum, 100 U per mL of penicillin G, 100 µg per mL of streptomycin sulfate, 0.25 µg per mL of amphotericin B as fungizone (Gibco-BRL, Grand Island, NY), 100 m*M* L-glutamine, and 5×10^{-5} M 2-mercaptoethanol (CRPMI) in 96-well flat-bottomed tissue culture grade plates at 37°C. Plates were then incubated with 1 µCu ^{3}H-thymidine (pulsed) for 24 hours, wells were harvested onto filter paper, and incorporated radioactivity was assessed by scintillation counting. For in vitro IgG production, plates were cultured with cells as above and were maintained for 18 days by replenishing CRPMI every 3 days. The plates were centrifuged at 300 × *g* for 5 minutes on Day 18 and the supernatant fluid was assessed for human IgG levels by enzyme-linked immunosorbent assay.

18-kDa CD154

The *Escherichia coli*-expressed 18-kDa CD154 was provided by Jean-Yves Bonnefoy, PhD (Glaxo IMB, Geneva, Switzerland) and is described in detail by Mazzei et al.[14] This molecule (18-kDa CD154) exists as an apparent homotrimer in solution.[14]

RESULTS

Four groups of Hu-PBL-SCID mice either were 1) not further manipulated (negative control group, Group 1), 2) received a single injection of 200 µg of 18-kDa CD154 (negative drug-treated control group, Group 2), 3) received twice weekly intraperitoneal challenge with HLA-A2 γ-radiated PBLs for 3 weeks, beginning on the day of reconstitution (positive control group, Group 3), or 4) were challenged as in Group 3 and received a single injection of 200 µg of 18-kDa CD154 on the day of reconstitution (Group 4). Mice were assessed for human IgG and IgM levels on Day 21 after reconstitution and all groups of mice made human IgG and IgM, which indicated that human PBLs were successfully engrafted in the mice (Table 1). The levels of IgG and IgM in Groups 2 and 4 were not significantly different from that in Groups 1 and 3, although the mice in Group 4 made significantly lower levels of IgM ($p<0.05$).

Because 18-kDa CD154 may have a direct positive as well as negative in vitro effect on B cells,[7] we wished to determine if 18-kDa CD154 could affect human IgG production in vitro. To accomplish this, the same PBLs as injected into the mice were also cultured to assess their ability to generate in vitro IgG antibody production. Although in vitro IgG production showed a marginal increase with allostimulation (Fig. 1, Group A vs. Group C), the addition of 18-kDa CD154 did not significantly alter IgG levels ($p>0.05$). Hence, the in vivo and in vitro data suggest that 18-kDa CD154 does not inhibit overall B-cell IgG antibody production.

Sera from Hu-PBL-SCID mice were examined for alloantibody by flow cytometry at each bleed (Fig. 2). Unchallenged Hu-PBL-SCID mice (Group 1) made only marginal levels of alloantibody. Hu-PBL-SCID mice that were challenged with HLA A2-positive stimulator cells (Group 3) made significant levels of alloantibody ($p<0.0001$), but this alloantibody production was abrogated if they were treated with 18-kDa CD154 (Group 4) ($p<0.02$). Cumulative data for all mice from three independent experiments is shown in Fig. 2.

To determine if 18-kDa CD154 nonspecifically inhibited secondary IgG production from memory B cells, we examined the IgG tetanus antibody levels from treated and untreated Hu-PBL-SCID mice. Figure 3 shows that, while stimulation of Hu-PBL-SCID mice with HLA-mismatched PBLs caused a slight increase in production of tetanus antibody starting on Day 10, the administration of 18-kDa CD154 did not significantly interfere with the anti-tetanus IgG levels in these four groups of mice ($p>0.05$). In all cases, the reactivity of anti-tetanus IgG in the mice remained well

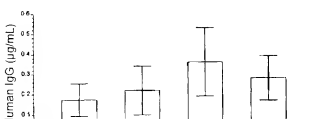

Fig. 1. Mean ± SEM concentrations of human IgG from PBLs cultured in vitro for 18 days. Unstimulated PBLs, Group A; unstimulated PBLs and 18-kDa CD154, Group B; PBLs stimulated with γ-radiated stimulator cells, Group C; PBLs stimulated with γ-radiated stimulator cells and 18-kDa CD154, Group D. No significant differences were observed between any of these groups.

TABLE 1. Human immunoglobulin levels (mg/mL) at Day 21 after engraftment*

	Unchallenged mice		HLA-challenged mice	
	IgG	IgM	IgG	IgM
Untreated	1.05 ± 0.19†	0.38 ± 0.04†	0.92 ± 0.14‡	0.42 ± 0.03‡§
18-kDa CD154-treated	1.27 ± 0.37‖	0.35 ± 0.09‖	0.95 ± 0.32¶	0.25 ± 0.045¶

* Mean ± SEM
† Group 1 mice
‡ Group 3 mice
§ $p<0.01$.
‖ Group 2 mice
¶ Group 4 mice.

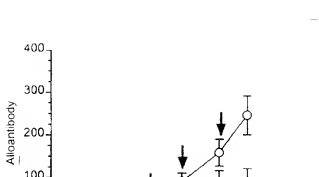

Fig. 2. Cumulative comparison of alloantibody responses over time as assessed by flow cytometry. Negative control Hu-PBL SCID mice were unchallenged and untreated (□ , n = 13 [Group 1]) as well as unchallenged and treated with 200 µg of 18-kDa CD154 on Day 0 (▽, n = 4 [Group 2]). Positive control mice were challenged with HLA-A2-positive PBLs (■ , n = 12 [Group 3]). We previously determined that these challenged mice make an alloantibody response specific for the HLA-A2 challenge antigen.[2] Test Hu-PBL-SCID mice were treated with 200 µg of 18-kDa CD154 on Day 0 and challenged with HLA-A2-positive PBLs (△, n = 12 [Group 4]) and assessed for IgG class alloantibody reactivity using HLA-A2-positive cells. The arrows denote the timing of the challenge PBLs. The difference between the challenged mice (O, Group 3) and 18-kDa CD154-treated challenged mice (△), Group 4) was significant, $p<0.02$. The data on the Y-axis is reported as the mean log fluorescence intensity (± SEM).

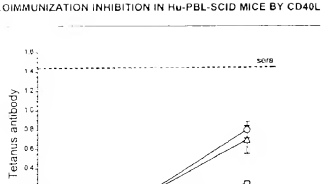

Fig. 3. Tetanus specific antibody production in HLA-A2-challenged, 18-kDa CD154-treated Hu-PBL-SCID mice. Hu-PBL-SCID mice (as in Fig. 2) were assessed for tetanus specific antibody. The difference between the challenged (■) and 18-kDa CD154-treated challenged (△) groups was not significant, $p>0.05$. The data on the Y-axis are reported as the mean absorbance (± SEM, n = 4 mice/group). - - -, levels of anti-tetanus from human donor sera taken at the time of reconstitution.

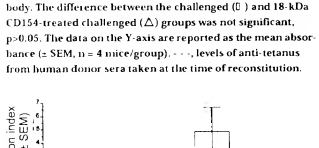

Fig. 4. Mean ± SEM stimulation index of a 4-day 1-way mixed lymphocyte culture. The stimulation index was calculated by dividing the cpm of A) unstimulated PBLs (responder cells); B) unstimulated PBLs and 18 kDa CD154 (responder cells and 18-kDa CD154); C) PBLs stimulated with γ-radiated stimulator cells (responder cells and stimulator cells); and D) PBLs stimulated with γ-radiated stimulator cells and 18-kDa CD154 (responder cells, stimulator cells, and 18-kDa CD154), by the cpm of responder cells alone.

below the levels of anti-tetanus from the donor's serum taken at the time of reconstitution (Fig. 3).

To determine if the administration of 18-kDa CD154 was also able to directly affect cell proliferation, responder cells (from the individual providing the PBLs for reconstitution) and stimulator cells (challenge cells) in the presence or absence of 18-kDa CD154 were set up as mixed lymphocyte cultures. The addition of γ-radiated stimulator cells to these cultures induced a 4.8-fold increase in cell proliferation (Fig 4). The addition of 18-kDa CD154 to the stimulator and responder cells in mixed lymphocyte culture prevented the increase in cell proliferation seen without 18-kDa CD154 (Fig. 4).

DISCUSSION

An Hu-PBL-SCID mouse model of a secondary alloimmune response was employed to establish the capacity of 18-kDa CD154 to act as a potential inhibitor of alloimmunization. Intraperitoneal inoculation of SCID mice with human PBLs resulted in engraftment in the mice, and challenge with HLA-mismatched PBLs resulted in specific alloantibody formation.

To determine if the administration of 18-kDa CD154 could reduce alloantibody levels, Hu-PBL-SCID mice were either treated with 18-kDa CD154 or not, and then they were challenged with HLA-mismatched γ-radiated PBLs. The administration of 18-kDa CD154 resulted in the inhibition of alloantibody formation. This inhibition was not 100 percent effective in our model, nor has it been 100-percent effective in models of allograft rejection.[20,21] However, in these models of allograft rejection, inhibiting the

CD40–CD154 pathway in conjunction with inhibiting the CD80/86–CD28 co-stimulatory pathway resulted in synergistically enhanced graft survival.[20,21] A synergistic immunomodulatory effect of inhibition by these co-stimulatory pathways has also been observed in a murine model of lupus, where the disease pathophysiology was attenuated by targeting of both pathways.[22]

To determine if IgG production in memory B cells was affected, in general, by 18-kDa CD154, we also examined the levels of anti-tetanus IgG in alloantigen-challenged SCID mice. The levels of IgG anti-tetanus were not different in 18-kDa CD154-treated and -untreated alloantigen challenge groups, which suggests that 18-kDa CD154 is not simply toxic to memory B cells.

The use of 18-kDa CD154 could inhibit alloantibody production by inhibiting T-cell function. To test this hypothesis, mixed lymphocyte cultures were performed and T-cell proliferation was shown to be reduced in the presence of 18-kDa CD154. Because B cells do not play a major role in the mixed lymphocyte culture, we speculate that T-cell activation and proliferation could be directly inhibited by 18-kDa CD154 treatment. Whether this T-cell inhibition is sufficient to explain the decreased alloantibody formation and whether that is due to a direct effect on T cells or to competitive antagonism of CD40 on APCs is at present unknown.

The alloimmune response to blood transfusion has been suggested to be a Th_2-dominant immune response.[23] Because CD154 is linked to T-cell activation along the Th_2 cytokine pathway,[14,24,25] and because T-cell proliferation can be defective when interleukin 4 is absent,[26] we speculate that 18-kDa CD154 may have decreased T-cell proliferation because it decreased Th_2-cytokine production. In addition, although helper T cells are the cells that undergo the proliferative response in the mixed lymphocyte reaction, competent APCs are also required for inducing optimal helper T-cell proliferation. APCs appear to benefit from T-cell priming, which increases their capacity to present antigens, and this priming appears to require proper CD40–CD154-dependent mechanisms.[27,28] It is possible that the 18-kDa CD154 used here somehow interacts with CD40 on APCs and either delivers to the APCs an incomplete signal that prevents priming or otherwise blocks endogenous T cells from priming the APCs via T-cell CD154.

We have thus demonstrated that the administration of 18-kDa CD154 can inhibit T-cell function and the alloimmune response. It is speculated that 18-kDa CD154 may have good therapeutic potential to inhibit human transfusion-induced alloimmunization.

ACKNOWLEDGMENTS

The authors thank the Canadian Red Cross Society blood donors whose participation made this study possible. They also gratefully acknowledge the important contribution made by Fred Ho, Janine Neville, Judy Burgess, and Margaret-Anne Crisell in donor recruitment, as well as that of the nursing staff at the Canadian Red Cross, Blood Transfusion Service, Toronto Centre. They also thank Stephanie In Sun Chung for performing the enzyme-linked immunosorbent assays.

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γ-Globulins prepared from sera of multiparous women bind anti-HLA antibodies and inhibit an established in vivo human alloimmune response

John W. Semple, Michael Kim, Alan H. Lazarus, and John Freedman

It has previously been shown that sera from multiparous women have increased levels of anti-idiotypic antibodies specific for anti-HLA molecules. γ-Globulins prepared from these sera may be superior to commercial preparations of intravenous γ-globulin (IVIg) for inhibiting HLA alloimmunization. To test this, $F(ab')_2$ fragments prepared from either commercial IVIg or from the sera of men or multiparous women were coupled to CNBr-Sepharose and tested for their ability to bind $F(ab')_2$ fragments derived from polyspecific anti-HLA sera. As determined by flow cytometry, compared with columns coated with $F(ab')_2$ derived from commercial IVIg or sera from men, columns coated with $F(ab')_2$ prepared from the sera of multiparous women bound significantly more anti-HLA. In addition, intact IgG molecules prepared from the sera of multiparous women significantly neutralized the reactivity of the anti-HLA $F(ab')_2$ fragments. To determine whether the intact IgG molecules or their corresponding $F(ab')_2$ fragments could affect in vivo alloimmunity, they were tested for their ability to inhibit an established IgG human alloimmune response in humanized severe combined immunodeficient (SCID) mice. Compared with commercial IVIg, when intact IgG or $F(ab')_2$ fragments derived from multiparous women were administered to SCID mice making human anti-HLA antibodies, a significant reduction in anti-HLA reactivity was observed. The findings suggest that IgG molecules prepared from the sera of multiparous women have increased anti-idiotypic reactivity against anti-HLA antibodies, which can significantly inhibit an established human IgG alloimmune response in an Fc-independent manner. (Blood. 2002;100:1055-1059)

Introduction

Intravenous γ-globulin (IVIg) is widely used to treat patients with immunoregulatory disorders, particularly chronic autoimmune thrombocytopenic purpura (AITP).[1-4] Although IVIg therapy has been shown to have a benefit in raising platelet counts in autoimmune platelet disorders, it appears to be not efficacious in patients with platelet-induced HLA alloimmunization.[5-8] The reasons for this are unclear but may relate to the nature of the immune response and the mechanisms of action of IVIg, that is, Fc receptor blockade/inhibition[8,9] or anti-idiotypic regulation.[10-12]

One of the mechanisms by which the peripheral antibody repertoire is regulated is via the production of antibodies reactive with the variable regions of other antibodies, that is, anti-idiotypes.[13-15] It has also been shown that the production and reactivity of anti-HLA antibodies are under the regulation of anti-idiotypic antibodies.[16-18] Perhaps the most striking example of natural in vivo anti-idiotypic regulation of alloimmunization to HLA is that associated with pregnancy, both at the level of the fetus and of the mother. Phelan et al[16] elegantly demonstrated that normal individuals contain anti-idiotypic antibodies to anti-HLA molecules exquisitely specific for the HLA antigens encoded from the noninherited maternal allele (NIMA), but not paternal HLA alleles. Additionally, anti-HLA reactivity may be transient in patients with malignancy,[20] and it has been shown that in these patients, as in pregnant women, anti-idiotypic antibodies may actively down-regulate anti-HLA antibodies.[21-24]

We speculated that pooling sera from multiparous women might increase the content of anti–HLA-specific anti-idiotypes to produce a more effective IVIg product for alloimmunized patients. We tested this by using affinity chromatography techniques combined with a humanized severe combined immunodeficiency (SCID) mouse model of alloimmunization. Our results indicate that, compared with commercial IVIg, IgG prepared from sera of multiparous women has higher anti-idiotypic binding capacity for anti-HLA and is superior to commercial IVIg or IgG prepared from men in inhibiting a secondary human alloimmune response.

Materials and methods

Sources and preparation of IgG

Three sources of IgG preparations were examined for anti–HLA-specific anti-idiotypic antibodies: (1) a commercial IVIg preparation (Immune Globulin Intravenous, 5%, Bayer, Etobicoke, ON, Canada), (2) the pooled sera of 34 never-transfused male volunteers (age range, 25-58 years), or (3) pooled sera from 47 multiparous women (age range, 27-47 years) who had their last pregnancy at least 1 year before blood sampling. Before pooling, anti-HLA reactivity in each serum was tested in a microlymphocytotoxicity (LCT) assay using a 30-cell panel of HLA typing cells (Canadian Blood Services, Toronto Center, Toronto, ON, Canada) and all were negative for anti-HLA antibodies. Equal amounts of serum from each

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individual were pooled. As a source of IgG anti-HLA antibodies, high-titered polyspecific anti-HLA human sera were obtained from the Canadian Blood Services (Dr B. Hannach, Toronto Center). For all 3 sources, IgG molecules were prepared by precipitation with 50% saturated ammonium sulfate followed by dialysis against 50 mM Tris-saline, pH 8.0. The IgG molecules were further purified by adsorption on QAE-Sepladex A-50 (Pharmacia, Mississauga, ON) to remove contaminating albumin.[25]

F(ab')$_2$ preparation

The F(ab')$_2$ fragments of the IgG molecules were prepared by standard methods to differentiate between idiotypic and Fc-mediated effects.[26] Briefly, the IgG molecules (1%-3% w/vol) were dialyzed against 0.2 M sodium acetate, pH 4.5, and digested with 2% (wt/wt) pepsin (Sigma, St Louis, MO) for 24 hours at 37°C. The F(ab')$_2$ fragments were then purified by Sephadex G150 (Pharmacia) gel filtration and protein G-Sepharose (Pharmacia) adsorption. Purity of the F(ab')$_2$ fragments was determined by high-performance liquid chromatography (HPLC) analysis using a Beckman Gold HPLC with an Altex TSK-3000 size exclusion column (1.5 × 30 cm) equilibrated in 0.05 M $NaPO_4$/0.1 M $NaSO_4$, pH 6.75. The final F(ab')$_2$ purity was typically more than 96%.

Anti-HLA neutralization assay

The 10-fold serial titrations (from 10^{-2} M) of intact IgG molecules from (1) commercial IVIg, (2) men, or (3) multiparous women were mixed with an equal volume (100 μL) of anti-HLA F(ab')$_2$ fragments (10^{-3} M final concentration) and incubated at 4°C for 18 hours. The mixtures were gently resuspended and 100 μL of protein A–conjugated Sepharose beads (Pharmacia) added for 45 minutes at 20°C to remove intact IgG. The beads were then centrifuged for 2 minutes at 800*g*, proteins in the supernatants quantified, and residual anti-HLA reactivity determined by flow cytometry.

Affinity chromatography

To study anti-idiotypic interactions, F(ab')$_2$ fragments derived from either commercial IVIg, the sera of men, or multiparous women were covalently coupled to CNBr Sepharose beads (Pharmacia) and 3.0-mL beads containing 60 mg coupled protein was poured into 1 × 8-cm glass chromatography columns (Pharmacia). Each column was extensively characterized with respect to baseline protein interactions. None of the columns could retain reactivity of a 50-μg load of a goat antihuman Fc-specific polyclonal antibody but did retain the reactivity from a 50-μg load of goat antihuman H+L chain–specific antibody. Additionally, none of the columns retained a 1-mg load of human albumin. For anti-idiotypic binding studies, all the columns were subjected to the same chromatographic protocol. One milligram of anti-HLA F(ab')$_2$ fragments (in 1 mL) was loaded onto the columns and allowed to continuously circulate at 0.5 mL/min for 18 hours at 4°C using a pump-driven closed loop system.[10] Columns were then washed with 20 column volumes of running buffer and the unbound protein collected. The columns were subsequently washed with 20 column volumes of 0.2 M glycine, pH 2.8, to elute bound proteins, which were then neutralized by adding 2 M Tris base (final concentration, 2.4% vol/vol). The eluted F(ab')$_2$ fragments were concentrated by membrane filtration. Both loaded and eluted proteins were made to the same concentration and anti-HLA reactivity was determined by flow cytometry.

SCID mouse model of human alloimmunization

Female CB.17 SCID mice (6-8 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME) and human alloimmunization was induced as previously described.[26] Briefly, human peripheral blood mononuclear cells (PBMCs) were obtained by 1 077 g/mL Percoll fractionation from a female blood donor with a history of prior pregnancy and low levels of circulating anti-HLA class 1 alloantibodies; she was blood group B$^+$, HLA A24/A34, B51/B62, Cw4, Bw4, Bw6 and had low but stable levels of circulating anti-HLA-B7 alloantibodies. The SCID mice were first engrafted with 1 × 10^7 of the donor's PBMCs and then challenged twice weekly for 4 weeks with 10^7 irradiated (2500 cGy) HLA A2/A2, Cw7$^+$ PBMCs from healthy laboratory volunteers. Anti-HLA antibody development was monitored weekly by flow cytometry. At the fourth week, if mice developed anti-HLA, they were randomized to receive 1 g/kg intraperitoneally of either intact IgG or F(ab')$_2$ fragments derived from commercial IVIg or from sera from men or from multiparous women, twice weekly for 4 weeks. These IgG dosages are similar to those used for human patients with AITP.[3] Serum anti-HLA reactivity after administration was compared with preadministration anti-HLA levels (week 4 of challenge).

Flow cytometry

For detection and characterization of anti-HLA, serial dilutions of the indicated test samples were incubated with 10^5 PBMCs from HLA-typed individuals for 45 minutes at 20°C and washed once. Fluorescein isothiocyanate (FITC)-conjugated goat antihuman IgG (H+L chain– or Fc-specific, Cedarlane Laboratories, Hornby, ON) was then added to the cells for 30 minutes at 20°C in the dark. Cells were analyzed by flow cytometry as previously described[24] using a FACSort flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser operating at 15 mW; 10 000 events were acquired through an electronic cellular gate set on lymphocytes based on forward and side scatter and were analyzed using LYSYS II software (Becton Dickinson).

Statistical analysis

Significance between means ± SD of the flow cytometric data was determined by Student unpaired *t* test for analysis of means.

Results

IgG derived from sera of multiparous women contains increased anti–HLA-specific anti-idiotypes

The intact IgG preparations were tested for their ability to neutralize the binding of anti-HLA antibodies. When titrations of intact IgG molecules derived from (1) commercial IVIg or from sera from (2) men or (3) multiparous women were incubated with F(ab')$_2$ fragments of anti-HLA, the IgG from multiparous women demonstrated significantly greater inhibition ($P < .01$) than was seen with IgG from either commercial IVIg or from men (Figure 1). To measure anti-HLA idiotypic binding, affinity columns coated with F(ab')$_2$ fragments derived from commercial IVIg, men, or multiparous women, were loaded with F(ab')$_2$ fragments made from anti-HLA sera and, after elution, the column-bound proteins were examined by flow cytometry. Compared with the anti-HLA

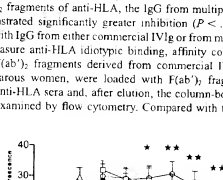

Figure 1 Inhibition of anti-HLA F(ab')$_2$ fragments. Ability of intact IgG derived from commercial IVIg (○), sera from men (□), or sera from multiparous women (●), to inhibit reactivity of anti HLA F(ab')$_2$ fragments. Anti-HLA reactivity is expressed as median channel fluorescence (mean ± SD, n = 6) at the indicated intact IgG/anti-HLA F(ab')$_2$ molar ratios. As controls, the anti-HLA reactivity of the 3 intact IgG preparations (x-axis = C) and of anti-HLA F(ab')$_2$ fragments (x-axis = 0, △) incubated only with phosphate-buffered saline is shown. The stars indicate significance (* $P < .05$, ** $P < .01$) between data points for intact IgG from multiparous women versus intact IgG derived from commercial IVIg.

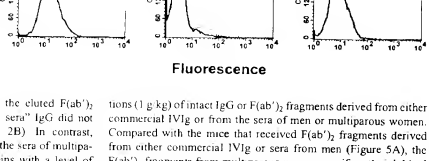

Figure 2. Flow cytometric histograms. Representative examples of flow cytometric histograms of the anti-HLA reactivities of 1 μg of (A) anti-HLA $F(ab')_2$ fragments loaded onto affinity columns, (B) protein eluates from the affinity columns coated with $F(ab')_2$ fragments from commercial IVIg ( ) or from sera of men ( ) and (C) protein eluates from the affinity columns coated with $F(ab')_2$ fragments derived from the sera of multiparous women.

reactivity of the loaded sample (Figure 2A), the eluted $F(ab')_2$ fragments derived from commercial or "male sera" IgG did not bind significant levels of anti-HLA (Figure 2B). In contrast, columns prepared using $F(ab')_2$ prepared from the sera of multiparous women (Figure 2C) yielded bound proteins with a level of anti-HLA reactivity approaching that seen with equivalent concentrations of loaded $F(ab')_2$ proteins (Figure 2A). Titrations of the eluted proteins showed that the affinity columns coated with $F(ab')_2$ made from the sera of multiparous women bound significantly higher amounts of anti-HLA than did columns coated with $F(ab')_2$ made from either commercial IVIg or sera from men ($P < .01$; Figure 3).

The LCT assays were used to determine the specificities of eluted anti-HLA antibodies. Antibodies of several anti-HLA specificities (HLA-A2, -A3, -A24, -A32, -B8, -B27, and -B39) were retained by the columns prepared from $F(ab')_2$ fragments from the sera of multiparous women, whereas only a weak anti–HLA-A2 reactivity could be detected in eluates from the columns prepared from either commercial IVIg or the sera of men.

Intact IgG and $F(ab')_2$ fragments derived from the sera of multiparous women can inhibit human alloimmunization

To determine the effect of intact IgG and corresponding $F(ab')_2$ fragments derived from multiparous women on a human alloimmune response, a SCID mouse model of human alloimmunization was used. When SCID mice were engrafted with PBMCs from an alloimmunized donor and challenged twice weekly with allogeneic PBMCs, human IgG anti-HLA antibodies developed in the sera of 93 of 102 (91%) mice by the second week of antigenic challenges. Figure 4 shows the level of reactivity of human IgG anti-HLA in the sera of the 93 SCID mice. At the fourth week of PBMC challenges, mice were randomized to receive twice weekly injections (1 g/kg) of intact IgG or $F(ab')_2$ fragments derived from either commercial IVIg or from the sera of men or multiparous women. Compared with the mice that received $F(ab')_2$ fragments derived from either commercial IVIg or sera from men (Figure 5A), the $F(ab')_2$ fragments from multiparous women significantly inhibited the reactivity of serum anti-HLA antibodies by the second week of administration (Figure 5B; $P < .01$). When intact IgG derived from either commercial IVIg or from sera of multiparous women was injected into alloimmunized (anti-HLA$^+$) SCID mice, a greater degree of inhibition was also observed compared with that seen with the corresponding $F(ab')_2$ fragments (Figure 5B versus 5A). Hence, IgG prepared from the sera of multiparous women was superior to IgG from commercial IVIg or the sera of men for the inhibition of an ongoing secondary anti-HLA immune response.

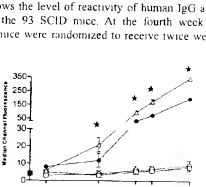

Figure 3. Anti-HLA reactivity. Anti-HLA reactivity (mean ± SD) of the median channel fluorescence values (n = 5) of the anti-HLA $F(ab')_2$ fragments loaded onto the affinity columns (△), and of bound proteins eluted from the affinity columns coated with $F(ab')_2$ derived from (1) commercial IVIg (○), (2) sera from males (□), or (3) sera from multiparous women (●). The median channel fluorescence of phosphate-buffered saline only is shown (at x-axis = 0 [■]). The stars indicate significance (★ $P < .01$) between data points comparing proteins eluted from the affinity columns coated with $F(ab')_2$ derived from commercial IVIg versus from sera of multiparous women.

Discussion

Therapy with IVIg is effective in treating immunodeficiency states, bacterial/viral infections, and immunoregulatory disorders, particularly immunohematologic disorders such as autoimmune thrombocytopenia, autoimmune neutropenia, and autoimmune hemolytic anemia.[12,27] Although the mechanisms of action of IVIg in immune regulation are complex and not yet fully elucidated, several theories have been postulated. In autoimmune thrombocytopenic disorders, for example, several experimentally supported theories of the mechanism of action of IVIg have been proposed. These include reticuloendothelial Fc receptor blockade,[4] down-regulation of FcγRIIa via FcγRIIb,[6] anti-idiotypic regulation,[10-12] and cytokine alterations.[3] In contrast to the recognized efficacy of IVIg therapy in *auto*immune disorders, there is controversy regarding its benefit in transfusion-induced HLA *allo*immunization.[3-8] Although several investigators have demonstrated that commercial IVIg preparations can inhibit anti-HLA in vitro,[28-35] the inhibition has been incomplete and may be the result of absence of the necessary

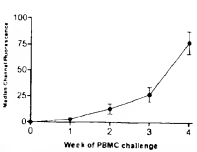

Figure 4. Anti-HLA alloimmune response in SCID mice. Development of human anti-HLA alloimmune response in SCID mice (n = 93) engrafted with PBMCs from an alloimmunized donor and challenged twice weekly for 4 weeks with allogeneic PBMCs. Data are expressed as the median channel fluorescence (mean ± SD) of SCID mouse sera at a 1:100 dilution.

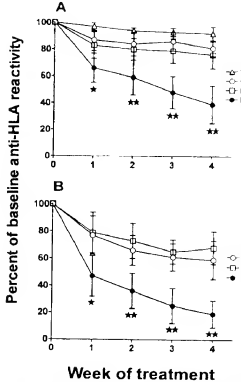

Figure 5. Inhibition of anti-HLA immunity in alloimmune SCID mice. Ability of (A) $F(ab')_2$ fragments or (B) intact IgG molecules to affect anti-HLA immunity in alloimmune SCID mice. The results of inhibition are expressed as the percent of baseline (100%) anti-HLA reactivity, that is, calculated from the median channel fluorescence values of each SCID mouse serum at each week after administration of proteins derived from commercial IVIg (○), the sera of men (□), or the sera of multiparous women (●), compared with the preadministration reactivity. The stars indicate significance (★, $P < .05$; ★★, $P < .01$) between data points (means ± SD) obtained using proteins derived from commercial IVIg versus the sera of multiparous women. The N values of each group of treated mice are as shown. Control mice that received no treatment with intact IgG or $F(ab')_2$ fragments are shown in panel A (△).

anti- HLA-specific anti-idiotypes in commercial IVIg.[36,37] Our results indicate that, compared with commercial IVIg or IgG prepared from the sera of men, the IgG derived from multiparous women has higher anti-idiotypic binding capacity for anti-HLA and can significantly inhibit an established human anti-HLA immune response in humanized SCID mice. Overall, these results support the hypothesis that IgG molecules prepared from multiparous women may be an effective γ-globulin product for the treatment of alloimmune platelet disorders.

Compared with the commercial IVIg or IgG derived from men, intact IgG molecules derived from multiparous women bound and neutralized significantly more anti-HLA $F(ab')_2$ fragments (Figure 1). Furthermore, compared with $F(ab')_2$-coupled affinity columns derived from either commercial IVIg or the sera from men, affinity columns coated with $F(ab')_2$ fragments from the sera of multiparous women bound significantly more anti-HLA in an idiotypic-dependent fashion (Figures 2 and 3). It is, however, important to note that IgG prepared from commercial IVIg or male donors was prepared in an identical fashion and used at the same concentration as the IgG derived from multiparous women; nonetheless, bound anti-HLA reactivity was different. The fact that IgG from a relatively small pool of male donors (n = 34) behaved similarly to the commercial IVIg (pooled from thousands of donors) suggests that pool size is not likely the reason for the lower anti-HLA binding capabilities (eg, due to dilution of anti-idiotypes on large-scale pooling). However, whether increasing the pool size of sera from multiparous women will change their anti-idiotypic binding patterns toward anti-HLA has not yet been established; we are currently studying this.

The probable explanation for higher anti-HLA idiotypic reactivity in the IgG preparations from multiparous women is likely because of the women's prior multiple exposures to paternal HLA antigens during pregnancy, resulting in anti-HLA alloimmunization and subsequent development of cross-reactive anti-HLA idotypes. This contention is supported, in part at least, by the observation that despite exposures to paternal HLA antigens, none of the sera collected after at least 1 year from the last pregnancy contained anti-HLA reactivity. Similarly, 94% of 109 multiparous women (different from those used in this study) screened by LCT had no detectible anti-HLA reactivity (B. Hannach, Canadian Blood Services, Toronto Center, personal communication). The need to screen the sera of multiparous women for anti-HLA reactivity before being used as a source of IgG production may be important because the presence of these antibodies may, when transfused, lead to the development of severe side effects such as transfusion-related acute lung injury (TRALI).[38] For example, it was recently demonstrated that administration of plasma from multiparous women to patients in intensive care units had a higher incidence of TRALI reactions compared to patients receiving control plasma units,[39] although donor plasma anti-HLA reactivity was not specifically determined.

To determine whether the IgG purified from the sera of multiparous women could inhibit an established human alloimmune response, we used a SCID mouse model of human alloimmunization.[26] When the $F(ab')_2$ preparations were administered to SCID mice already anti-HLA alloimmunized, $F(ab')_2$ preparations derived from the sera of multiparous women significantly inhibited SCID mouse serum anti-HLA reactivity. This supports the conclusions based on the affinity chromatography experiments that sera from multiparous women contain more anti-HLA-specific anti-idiotypes and suggests that these anti-idiotypes can more effectively inhibit a human anti-HLA response than does commercial IVIg. The time kinetics of in vivo HLA alloimmune inhibition within the first week after the initial $F(ab')_2$ administration have not been determined; however, the data are in agreement with those reported in several transplant studies showing that anti–HLA-specific anti-idiotypes are correlated with lower alloimmunization rates.[16-18]

Intact IgG preparations from multiparous women or commercial IVIg caused a greater inhibition of anti-HLA reactivity in the SCID mice than did their corresponding $F(ab')_2$ fragments (Figure 5). Although this may suggest that the presence of the Fc region has an additive effect to anti-idiotypic regulation in reducing HLA alloimmunization, it could not be ruled out that the differences may be also due to different in vivo half-lives of intact IgG and $F(ab')_2$ fragments in this mouse model.

Studies in several animal models have recently shown that the mechanism of IVIg in the reversal of either autoimmune- or xenoimmune-mediated thrombocytopenia is primarily due to Fc-dependent inhibition of the reticuloendothelial system.[9,40,41] Our results suggest that, with respect to HLA alloimmunization, an IgG product can be produced from the sera of multiparous women that mediates its effects via anti-idiotypic interactions. These apparent discrepant results underscore that γ-globulins may have multiple mechanisms of action, which may be reflected by differing methodologies or type of immune phenomenon studied.

In summary, sera from multiparous women have an increased content of anti-idiotypic antibodies specific for anti-HLA alloantibodies. These purified anti-idiotypic antibodies can significantly inhibit an established IgG anti-HLA immune response in a humanized SCID mouse model. The results suggest a new and relatively simple approach to producing a superior γ-globulin product for the treatment of platelet alloimmunization.

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Human- SCID mouse chimeric models for the evaluation of anti-cancer therapies

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The ability to engraft human tumors and human immunocompetent cells successfully in severe combined immunodeficient (SCID) mice has spawned the development and use of human- mouse chimeric models to evaluate anti-cancer therapies. The lack of standardization and many other potential pitfalls have contributed to the current controversy surrounding the reliability of these different models. Five frequently used SCID mouse models and their specific applications are summarized with the specific aim of providing an objective discussion of the strengths and limitations of each model, together with suggestions for overcoming some of the variabilities and for improving the design and use of future models.

Since the first report indicating that severe combined immunodeficient (SCID) mice could be successfully engrafted with human tumors[1], a wide variety of solid human tumors and hematological neoplasms, both as cell lines and as fresh biopsy tissues, have been engrafted into SCID mice[2]. Shortly after the first successful engraftment of human tumors, human peripheral blood leukocytes (HuPBLs) were shown to engraft following their inoculation into the peritoneal cavity of SCID mice[3]. These early observations led to the development of human–SCID mouse chimeric models, which have been exploited successfully to evaluate a wide variety of anti-cancer therapies. Five of the most frequently used human- SCID mouse chimeric models are summarized, with their specific applications and limitations, in Table 1. The first four models involve the use of human tumor-cell lines to establish xenografts in SCID mice. In the fifth model, the xenografts are generated by engrafting nondisrupted pieces of human tumor biopsy tissue. A better understanding and appreciation of both the potential and limitations of each of these models requires some knowledge of the basic biology of the SCID mouse, as well as the biological consequences of xenografting human tissues into these mice.

The limitations of human- SCID mouse chimeric models and their possible solutions

The SCID mouse arose initially from a point mutation in chromosome 16 in the CB-17 inbred mouse strain[4]. This mutation, which reflects a more generalized defect in DNA repair[5], results in the interruption of lymphocyte maturation and a deficit in circulating, mature, functional T and B-cells. In contrast to their lack of functional adaptive immunity, SCID mice possess a completely intact innate immune system, with normal numbers of monocytes/macrophages, natural killer (NK) cells and granulocytes[6,7].

It is important to recognize that, immediately following the inoculation of human tissues (both tumors and leukocytes), SCID mice mount a complex, multistage immune response against the human xenograft, involving innate immune cells and soluble factors. The intensity of this host-versus-graft disease (HVGD) response can vary considerably from mouse to mouse, and has also been shown to vary with the histological type of tumor used for engraftment[8]. Following intraperitoneal (i.p.) engraftment of human cells, there is a dramatic increase in murine leukopoiesis, massive neutrophil recruitment into the site of the engraftment and the induction of expression of several murine cytokines, including interleukin-1β (IL-1β), IL-4, IL-6, IL-10, IL-12, tumor necrosis factor (TNF), interferon α (IFN-α), IFN-β and IFN-γ, which are detected as early as one day post engraftment and persist for up to two weeks after the transplant[8,9]. Murine granulocytes[8,9], macrophages[10] and NK cells[11] have all been shown to contribute to the inhibition of human-cell engraftment.

The reactions of the SCID mouse to human tissue xenografts must be fully recognized and defined for two reasons. First, these reactions must be taken into consideration when attempting to interpret results obtained using any of the chimeric models listed in Table 1 accurately. Second, the identification of the murine effector cells and cytokines that are responsible for inhibiting the engraftment of human cells has made it possible to improve the success of human leukocyte and tumor xenografts significantly by selectively eliminating or blocking the anti-graft activities of both the murine cells and their soluble products[8,10,11]. These approaches can considerably decrease the variability observed in the human–SCID mouse chimeric models.

Xenograft-versus-host disease (XGVHD) reactions are another potential pitfall when human immunocompetent cells are engrafted into SCID mice; for example, during i.p. inoculation of HuPBLs (Model Two), which recognize and respond vigorously to murine tissue antigens. This xenoresponse, mediated by human B and T-cells, not only causes potentially fatal XGVHD, but also severely limits the ability of HuPBLs to respond to exogenous antigens[12,13]. The problem has been minimized by co-injecting HuPBLs

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Table 1. Human–SCID mouse chimeric models for the evaluation of anti-cancer therapies[a]

Model	Engrafted tissue[b]	Applications	Limitations[c]									
			XGVHD	HVGD	EBV lymphoma	Lacks micro-environment	Histo-incompati-bility	Patient tissue	Spontaneous tumor development	Cell lines	DNA repair defect	Refs
One: HuTum–SCID	Human tumor cell lines	Orthotopic/ metastases Drug delivery and/or therapy (Cytotoxic and/or antiangiogenic drugs; mAb-directed)	No	Yes	No	Yes	No	No	Yes	Yes	Yes	9–12,38, 41, 47–53
Two: HuTum × HuPBL–SCID	Human tumor cell lines plus human PBLs	Immunotherapy (active and passive) Identify effector cells	Yes[d]	Yes	Yes	Yes	Yes[e]	Yes	Yes	Yes	No	14–16, 26–28
Three: HuTum × effector–SCID	Human tumor cell lines plus CTLs, LAKs, DCs, macrophages and NK cells	Immunotherapy (passive) Adoptive cell therapy	No	Yes	No	Yes	Yes[f]	No	Yes	Yes	No	20, 42–46
Four: EBV HuPBL –lymph–SCID	Human PBLs from EBV seropositive donors	Lymphoma-genesis Immunotherapy (passive)	Yes	Yes	N/A	Yes	No	No	Yes	No	No	3,18,19, 33,34
Five: HuTum × HuStroma–SCID	Intact patient tumor biopsy tissue or human tumor cells and fetal stroma	Tumor–stroma interaction Immunotherapy (active and passive) Assess TIL function and the role of the microenvironment	No[g]	Yes	No[g]	No	No[g]	Yes	Yes	No[h]	No	12,54, 59,60, 63,64

[a]Abbreviations: CTL, cytotoxic T lymphocyte; DC, dendritic cell; EBV, Epstein–Barr virus; HuPBL, human peripheral blood leukocyte; HuTum, human tumor; HVGD, host-versus-graft disease; i.p., intraperitoneal; i.v., intravenous; LAK, lymphokine-activated killer; mAb, monoclonal antibody; N/A, not applicable; NK, natural killer; s.c., subcutaneous; SCID, severe combined immunodeficient; TIL, tumor infiltrating leukocyte; XGVHD, xenograft-versus-host disease.

[b]HuPBLs and effector cells have been engrafted by i.p., s.c. or i.v. means. The tumor cell lines have been inoculated by i.p., s.c. or i.v. means or into the organ from which they originated (i.e. orthotopically). Fresh tumor biopsy tissue is surgically implanted as intact pieces (s.c.) and fetal stroma is engrafted s.c.

[c]XGVHD is caused by the response of HuPBLs to mouse tissue antigens; HVGD is the response of murine innate effector cells to human tissues. EBV is responsible for the development of spontaneous B cell lymphomas from within the pool of human B cells present in HuPBL xenografts. Other limitations include: the lack of a human tumor microenvironment; the requirement for fresh patient tissue, which increases the logistical difficulty of the model; the spontaneous generation of murine thymomas that occur in ~10% of SCID mice by nine months of age; the use of human cell lines (both tumor and effector), which reduces the physiological relevance of the model; and the fact that the SCID mutation involves a defect in DNA repair making mice more susceptible to the toxic effects of radiation and many DNA-damaging cytotoxic drugs.

[d]Human immunocompetent cells placed s.c. rarely lead to a XGVHD. However, when placed i.p., XGVHD is a significant problem.

[e]EBV positive lymphomas generated from the TILs in the tumor xenograft are observed with <5% of the xenografts established with fresh lung tumor biopsy tissue.

[f]These models most often employ HuPBLs or effector cells that are allogeneic with respect to the tumor, but it is possible to avoid the histoincompatibility by using immunocompetent cells and tumor from the same patient (i.e. autologous embodiment).

[g]The histoincompatibility only occurs when one uses fetal tissues as surrogate microenvironments.

[h]Human tumor cell lines are sometimes engrafted in conjunction with fetal tissue.

with tumors subcutaneously (s.c.) instead of i.p. Thus, the HuPBLs are restricted from migrating and encounter fewer xenoantigens[14]. Although the s.c. implantation of HuPBLs and tumor minimizes the problems of XGVHD and the paralysis of the response of HuPBLs to exogenous (nonmurine) antigens, only the i.p. inoculation of HuPBLs permits the testing of strategies that are designed to induce and expand an adaptive immune response with a memory component (e.g. vaccination strategies). It has now been established that the anti-mouse immune response of HuPBLs inoculated i.p. can be inhibited by preventing CD40–CD40 ligand (CD40L) costimulation[15]. Others have also shown in a SCID mouse model that the blockade of the CD40–CD40L costimulatory pathway inhibits the reactivity of HuPBLs to MHC differences[16]. These findings suggest that the problem of XGVHD in Model Two might be reduced or completely eliminated by inhibiting human CD40–CD40L interaction in the presence of mouse antigens.

Engraftment of HuPBLs from Epstein–Barr virus (EBV) seropositive donors results in the spontaneous

development of human B-cell lymphomas in SCID mice[3]. This represents a potential pitfall of nearly all uses of Model Two, and protocols that are designed to enhance or promote the engraftment of the HuPBL s also potentiate the development of EBV-induced lymphomas[17]. A better understanding of the mechanisms involved in lymphomagenesis should lead to a means to circumvent this problem in the chimeric human–SCID mouse model. The spontaneous development of the EBV$^+$ lymphomas, which represents a pitfall of Model Two, has actually been successfully exploited in Model Four to study the mechanisms of lymphomagenesis in humans[18,19].

During the past ten years there have been >500 papers published on the use of the first four SCID mouse models to evaluate cancer therapy. Specific examples have been selected and summarized below to illustrate the flexibility of the SCID mouse models as a preclinical tool for testing novel anti cancer therapies. It will be clear from these summaries that, although none of the four SCID models completely recapitulates the biology of the tumor and immune system in patients, these models have provided valuable insights with respect to therapeutic effects beyond that possible with current *in vitro* methods or nonSCID animal tumor models. Model Five, in which the response of human inflammatory cells to tumors can be studied within the tumor microenvironment, is discussed separately.

The evaluation of immune-based cancer therapies

Cytokine- and chemokine-based immunotherapies

Model One has been utilized to evaluate cytokine and chemokine therapy by taking advantage of the fact that SCID mice possess functional NK cells, macrophages and polymorphonuclear cells that can be activated by both human and mouse cytokines to suppress the growth of human tumor xenografts. Although this is a contrived system, it provides a simple initial screen for evaluating cytokine-based strategies. Cytokines that have been tested in this model include IL-2, IL-10, IL-12, IL-18 and Flt3 ligand, which were shown to induce their anti-tumor effects primarily through murine NK cells[20–25]. Serious limitations of this model include the xenogeneic nature of the anti tumor effector cells and the inability to evaluate adaptive immunity.

Model Two provides an opportunity to evaluate human cytokines and chemokines that augment both innate and adaptive anti-tumor immune responses of human leukocytes, thus providing a model that is more clinically relevant. This model has been utilized to test the anti-tumor efficacy of a wide spectrum of human cytokines[14,26,27] and cytokine-delivery strategies, including the direct injection of soluble cytokines[28], cytokine gene transfer[26,27] and the use of slow release polymer particles (biodegradable microspheres)[28]. In the overwhelming majority of these studies, HuPBLs were allogeneic with respect to the tumor and the allo-anti-tumor response was found to be mediated primarily by CD8$^+$ T and CD56$^+$ NK cells. In one study, a similar augmentation of an autologous anti-tumor immunity was also demonstrated[28].

Antibody-based therapies

(1) Anti-tumor antibodies. The simplest antibody (Ab)-based therapy involves the treatment of SCID mice bearing human tumor xenografts with systemic injections of monoclonal antibodies (mAbs) directed against molecules expressed by the tumor cells. The mechanism responsible for the anti-tumor effect observed in these systems is Ab-dependent cellular cytotoxicity (ADCC), mediated either by resident host cells[29], adoptively transferred human effector cells [HuPBLs and lymphokine-activated killer cells (LAKs)][30,31] or a combination of both. Of special note is the use of anti-human CD40 Abs for the treatment of human B-cell lymphoma xenografts, because ligation of CD40 on lymphoma cells has direct anti-proliferative effects, in addition to mediating ADCC (Ref. 32). Anti-CD40 Ab treatment has also been shown to inhibit lymphomagenesis in Model Four[33,34].

(2) Immunocytokines. Ab–cytokine fusion proteins (immunocytokines) have been tested in SCID mice with established human tumor metastases. Targeting of either human IL-2 (Ref. 35) or human IL-12 (Ref. 36) to the site of the tumor inhibited the growth of metastatic human tumor cells and extended the survival time of animals with xenografts. In all of these studies, SCID mice were reconstituted with human LAK cells, which were required for the anti-tumor effect of IL-12 immunocytokine therapy[36]. However, the anti-tumor efficacy of Ab–IL-2 fusion therapy was not dependent on human LAKs and was mediated by murine macrophages[37].

(3) Immunotoxins and immunoliposomes. Numerous immunotoxins targeting leukocyte surface antigens have been tested in Model One for the treatment of various human hematological malignancies engrafted in SCID mice[38]. These models have provided justification for the clinical evaluation of several of these immunotoxin compounds. A novel human skin xenograft–SCID mouse model for testing the effects of immunotoxins on vascular leakage has recently been reported and could be useful for determining the effect of different immunotoxins on vascular leakage in humans[39].

The targeted delivery of chemotherapeutic drugs encapsulated in immunoliposomes is another area in which Model One has been utilized successfully. The anti-tumor efficacy of drug-containing Ab-conjugated liposomes that target human lymphoma, breast and lung tumor xenografts in SCID mice has been tested[40,41]. These studies have shown that immunoliposomes represent a superior alternative to free drug or drug-encapsulated nontargeted liposomes for the suppression of established primary tumors and the prevention of metastasis[40,41].

A serious limitation of all studies of Ab-based therapy in SCID mouse models of cancer is the lack of

normal human tissue to assess Ab cross-reactivity and toxicity. In addition, the SCID mouse host does not generate an immune response against the therapeutic Ab as would be expected in immunocompetent human patients. When assessing the results of Ab therapies in the SCID mouse models, it is important to take into consideration the isotype of the Ab, whether the intact Ab or an Ab fragment was used, which murine cells were depleted and whether human effector cells were added.

Assessment of effector cells

One of the earliest successful uses of Model Three established that human cytotoxic T lymphocytes (CTLs) were able to suppress the growth and spontaneous metastasis of human melanomas[42]. The suppression of both primary tumor recurrence after surgical resection and tumor metastasis illustrated the ability of the adoptively transferred CTLs to home to and suppress tumor growth *in vivo*. It was further established that exogenous IL-2 enhanced the CTL-mediated anti-tumor immunity[42]. Whenever human cytokines known to be biologically active on murine cells (e.g. IL-2) are used, it is important to establish whether murine cells are contributing to the anti-tumor effect. In this particular case, the authors ruled out the possible contribution of SCID mouse NK and LAK cells[42]. Unfortunately, many studies have failed to address the role of host effector cells and cytokines in mediating the outcome of selected therapies.

Model Three has also been used to test the ability of Abs to direct the delivery of LAK cells to human tumor xenografts[43]. In these studies, LAK cells alone did not inhibit the growth of tumor xenografts, but the addition of a bispecific Ab increased the uptake of LAK cells into the tumor xenograft, resulting in decreased tumor growth and increased survival of the mice[44].

Additional studies have confirmed that both allogeneic[44,45] and autologous[46] human tumor-specific CTLs can suppress the growth of several different tumor types. All of these studies showed some tumor suppression, but differences in the level of suppression and the requirement for cytokines were noted. Some of these differences are probably owing to variations in the protocols used for engraftment and treatment.

The evaluation of nonimmune-based cancer therapies

The SCID mouse models (particularly Models One to Three) have been used effectively to evaluate novel chemotherapeutic drugs and different drug-delivery systems. The results of these studies suggest that the data obtained from SCID mouse models correlate with observations in cancer patients, thus, the SCID mouse models might be useful for *in vivo* testing of new agents and methods of drug delivery[47]. For example, a clear superiority of sterically stabilized liposomes over conventional liposomes for the delivery of doxorubicin to ectopically placed lung tumor xenografts was established using Model One[48,49]. By transfecting lung tumors with a tumor marker gene and monitoring tumor progression by quantifying the level of the marker in the serum of SCID mice[50], it has been possible to evaluate the effects of chemotherapy using different drug-delivery systems upon the growth and metastasis of tumor xenografts placed orthotopically in the lungs of SCID mice[41].

The use of SCID mouse models to study human tumors within a human microenvironment

One of the major limitations of the first four SCID models is the lack of a human microenvironment, which is now known to play a crucial role in regulating tumor growth and metastasis, and in the response to immunotherapy. New model systems (Model Five, Table 1) have been developed that allow human tumor cells to be engrafted into SCID mice in the context of a human microenvironment.

Surrogate human tumor microenvironments

Initial attempts to engraft human cancer cells in the context of a human microenvironment involved a two-step process whereby SCID mice were first engrafted with normal human tissue, primarily derived from fetal organs (e.g. thymus, bone marrow, spleen, liver, lung and skin), followed by direct inoculation of human cancer cells into the fetal xenograft[34–36]. In this system, the fetal tissue functions as a surrogate human microenvironment within the murine host. For example, human fetal thymus xenografts have been shown to support the growth of fresh cells from human T cell non-Hodgkin's lymphoma patients[34]. Similar models have been used to study the *in vivo* metastasis of human myeloma[36], lung cancer[37] and colon cancer[38] cell lines to human tissue established in SCID mice.

Natural human tumor microenvironments

Engraftment of SCID mice with histologically intact fragments of human tumor tissue provides a less-contrived method for studying human neoplasms in the context of an autologous tumor microenvironment (Fig. 1). This approach offers several advantages over the use of fetal-tissue surrogate microenvironments. For example, the tumor and stromal microenvironment are from the same individual and thus histocompatibility differences are not a concern. In addition, many of the elements of a 'natural' tumor stroma will be present within the microenvironment, including tumor-associated extracellular matrix (ECM), vasculature and human tumor-infiltrating leukocytes (HuTILs), which are probably tumor-specific and tumor-reactive (Fig. 1). Because the tissue is surgically implanted into SCID mice with minimal *in vitro* manipulation, the original local architecture of the tumor specimen is not disturbed and can be maintained in the xenograft for up to 22 weeks[59].

Several human malignancies have been studied by engrafting nondisrupted patient tumor tissue into SCID mice, including tumors of the lung[12,59–61], skin[62], prostate[63], breast[64] and thyroid[65]. The first report,

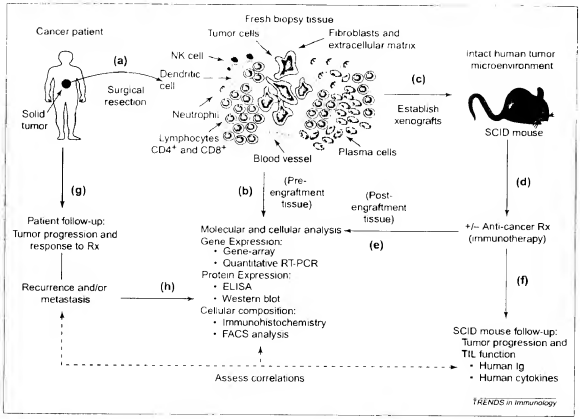

Fig. 1. Model Five: monitoring the effects of anti-cancer therapy on patient tumor xenografts. (**a**) Following surgical resection of a solid neoplasm, a piece of the fresh tumor biopsy tissue (i.e. pre-engraftment) is saved for (**b**) molecular and cellular analyses, and (**c**) the remaining tissue is engrafted as histologically intact tumor fragments into the subcutis of natural killer (NK) cell-depleted severe combined immunodeficient (SCID) mice. Each patient's tumor is engrafted into 10–40 mice, establishing xenografts with an intact tumor microenvironment including both tumor and tumor-infiltrating lymphocytes (TILs). (**d**) One week after engraftment, mice receive anti-cancer treatment (Rx) or control treatment given either intra-tumorally or systemically. (**e**) At different time-points post-treatment, the treated xenografts are biopsied by fine needle aspiration (FNA), or the xenograft is completely removed from the mouse for analysis. Gene expression is analyzed by gene-array and quantitative reverse transcriptase (RT)-PCR of tumor-derived RNA; protein expression is assessed by enzyme-linked immunosorbent assay (ELISA) and western blots of tumor lysates, and the cellular composition of the xenografts is determined by immunohistochemistry and fluorescence-activated cell sorter (FACS) analysis. (**f**) The *in vivo* growth of tumor xenografts is monitored by weekly tumor measurements, and human TIL (HuTIL) function is assessed by quantifying the level of human Ig and human cytokines [e.g. interferon-γ (IFN-γ)] in SCID mouse sera. The extent of tumor metastasis in SCID mice is determined by necropsy when the experiment is terminated. (**g**) The patient whose tumor was xenografted is followed clinically for tumor progression and response to therapy. (**h**) In the event of a tumor recurrence or metastasis, attempts are made to obtain a second biopsy sample for analysis and/or xenografting into additional SCID mice. Correlations among tumor progression in the patient, the cellular and molecular analyses of both the pre-engraftment tumor specimen and tumor xenografts, and tumor progression in SCID mice are assessed.

published in 1989, indicated that subcutaneous implantation of intact human lung tumor specimens into SCID mice resulted in the successful engraftment of both tumor and TILs (Ref. 12). The histology of the xenografts and the presence of human Ig (HuIg) in the sera of xenografted mice served as indicators of HuTIL engraftment in this study[12]. In another early study, Charley *et al.* transplanted lesional skin from a patient with cutaneous T-cell lymphoma and monitored the graft for human CD4+ leukemia cells. The human leukemic cells not only persisted within the lesional-skin graft for a period of one month after transplantation, but the xenografted skin maintained the original histopathological features observed in the patient, suggesting that the human tumor microenvironment was preserved following engraftment into SCID mice[62].

The surgical implantation of nondisrupted human lung tumor tissue (Fig. 1) has revealed the following: (1) successful engraftment of HuTILs occurs in >95% of xenografted patient tumors, regardless of the histological type of tumor[59]; (2) HuTILs remain at the site of the xenograft and show evidence of proliferation *in situ*[59]; (3) the HuIg present in the SCID mouse sera is primarily IgG (Ref. 61) and is produced by human plasma cells in a T-cell-dependent manner[59]; (4) sera from xenografted mice contain Abs that react against proteins derived from autologous tumor tissue[59,61] as well as allogeneic lung tumor cell lines[61], but not normal lung tissue[61]; (5) high levels of HuIg in the sera of mice engrafted with tumor biopsy tissue are associated with the growth arrest of adenocarcinoma xenografts[59], and (6) serial passage of lung tumor xenografts in SCID mice results in the loss of HuTILs from the tumor microenvironment and the disappearance of HuIg from the SCID mouse sera[59].

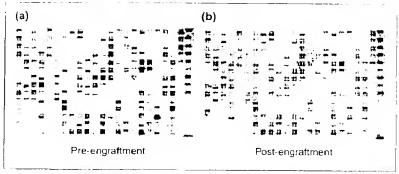

Fig. 2. Gene expression patterns in (a) an original patient tumor biopsy specimen and (b) the tumor xenograft nine days after engraftment. The expression of ~375 different human genes (including those encoding cytokines, chemokines, cell-surface molecules, growth factors, proteases, angiogenic factors and tumor markers) was analyzed by gene-array technology. Briefly, intact fragments of human lung tumor tissue were implanted subcutaneously (s.c.) into severe combined immunodeficient (SCID) mice to establish xenografts. Tissue from the original specimen (i.e. pre-engraftment) was also saved for RNA isolation. Nine days after engraftment, the untreated xenografts were removed from mice and total RNA was prepared from both the pre-engraftment tissue and post-engraftment tissue samples. Radiolabeled (^{33}P) cDNA was synthesized from tumor-derived RNA and then hybridized overnight to Human Cytokine Expression Macroarrays (R & D Systems, Minneapolis, MN, USA). Following exposure to a low-energy phosphor screen, images were generated using the STORM 860 Phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Note that the expression of most genes (spotted in duplicate pairs on the macroarray) is similar between (a) pre-engraftment tissue and (b) post-engraftment tissue, indicating that: (1) the tumor microenvironment is functionally preserved even after nine days in the SCID mouse; and (2) exposure of human tumor tissue to the murine environment has not significantly altered gene expression patterns within the patient's tumor.

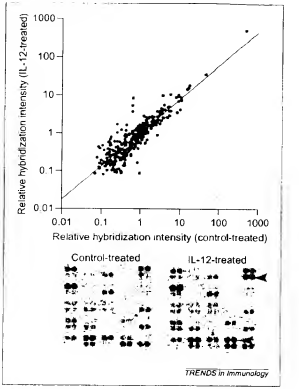

Fig. 3. Data obtained from gene-array analysis of human lung tumor xenografts following treatment *in vivo*. Xenografts were established subcutaneously (s.c.) in severe combined immunodeficient (SCID) mice from a patient lung tumor biopsy specimen, and treated one week later by direct intra-tumoral injection of biodegradable microspheres loaded with recombinant human interleukin-12 (rhIL-12), or control (cytokine-free) microspheres. Two days after treatment, xenografts were removed for isolation of total RNA. Gene-array analysis was performed as described in Fig. 2. The average background value was subtracted from the signal of each spot. Signals were then normalized by correcting for the total signal intensity of all the spots on the arrays. The normalized, average signal intensities from each gene expressed in both the control-treated xenograft and the IL-12-treated xenograft were plotted against one another. Plots that lie above the regression line represent genes more highly expressed in the IL-12-treated xenograft, and plots below the line indicate genes more highly expressed in the control-treated xenograft. Note that the majority of the >200 expressed genes are not affected by injection of human IL-12 into the xenograft. However, the expression of a number of genes is either increased or decreased as a result of IL-12 treatment. A selected region of the macroarray from IL-12-treated and control-treated xenografts is shown. The red arrowheads mark the location of two genes, the expression of which was significantly enhanced by IL-12 treatment.

More recently, it was shown that HuTILs are able to limit the growth of human lung tumor xenografts in SCID mice[60]. This TIL-mediated tumor suppression was caused, at least in part, by the production of endogenous human IL-12, because treatment of mice with neutralizing Abs to human IL-12 resulted in the enhanced growth of primary human lung tumor xenografts[60]. The addition of exogenous human IL-12, either by genetically modified cells or by cytokine-loaded biodegradable microspheres, augmented the anti-tumor activity of HuTILs (Ref. 60) in this model. Additional experiments revealed that recombinant human (rh)IL-12 stimulated the TIL-dependent elevation of human IFN-γ serum levels, which correlated directly with the degree of tumor suppression observed.

To gain further insight into the molecular events taking place within the microenvironment of lung tumor xenografts, global gene expression patterns were analyzed in both the pre-engraftment tumor tissue and the tumor xenografts before and after treatment with cytokines, using gene-array technology. As illustrated in Fig. 2, the pattern of gene expression in an untreated xenograft nine days after tumor engraftment was nearly identical to that seen in the original patient tumor (i.e. pre-engraftment). This established that gene expression within human tumor tissue was not significantly altered following engraftment into SCID mice. By contrast, treatment of the same patient's tumor xenograft with rhIL-12 altered the expression of several genes, including those encoding cytokines, chemokines, adhesion molecules and angiogenic factors (Fig. 3). As predicted from the increase in human IFN-γ levels seen after rhIL-12 treatment, increases in the IFN-γ-inducible genes encoding monokine induced by IFN-γ (MIG), IFN-inducible protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), inducible nitric oxide synthase (iNOS), β_2-microglobulin (β_2-m) and HLA were all detected by macroarray analysis. Decreases in the levels of vascular endothelial growth factor (VEGF), platelet endothelial cell-adhesion molecule (PECAM), Gro-α and several markers of human lung cancer [for example, extracellular matrix metalloproteinase inducer (EMMPRIN), the proteins midkine and ephrin, and integrins-α3 and -α6] were also observed after rhIL-12 treatment.

Collectively, these results suggest that, following the implantation of fresh tumor tissue, the human

inflammatory cells (i.e. TILs) maintained within the tumor microenvironment remain functional and responsive to exogenous cytokine stimulation, but are largely nonresponsive to host tissue antigens (Fig. 2). More importantly, predicted changes in gene and protein expression are observed following cytokine treatment of tumor xenografts (Fig. 3) that can be correlated with a suppression of tumor growth in SCID mice[60]. Because this model results in the co-engraftment of tumor and anti-tumor effector cells within the native tumor microenvironment, it is now possible to study the response of the autologous HuTILs *in situ* to different therapeutic strategies, while evaluating the effects of therapy on tumor progression *in vivo* (Fig. 1).

The use of fine-needle aspirates (FNAs)[66] to biopsy the tumor xenografts periodically without removing them from the SCID mouse host has extended the utility of this model. It has recently been demonstrated that sufficient quantities of RNA can be obtained from the FNAs for both reverse transcriptase (RT)-PCR and gene-array analyses, thereby providing the opportunity to monitor changes in gene expression (Figs 2,3) without sacrificing the mice. As the mice are not sacrificed, any changes in gene expression within the xenografts can be correlated with subsequent changes in tumor growth or metastasis.

Future directions

For each of the models discussed, remedial protocols, such as elimination of host effector cells and different engraftment protocols, must be standardized. Particular attention must also be given to standardizing the routes, doses and schedules of inoculation of human cells (e.g. fresh tumor tissue, tumor cell lines and leukocytes), and to the administration of therapeutic agents (e.g. drugs, cytokines, effector cells and Abs). This will make it possible to compare results from one laboratory to another reliably.

Despite attempts to standardize protocols, some variability will be beyond the control of the investigator, especially when fresh human clinical specimens are engrafted (i.e. Model Five). Inherent differences among patient tumors, with respect to proliferation, metastatic potential, capacity for angiogenesis and the degree of TIL infiltration and function, should be expected. A more rigorous analysis of the tumor xenograft will make it possible to recognize and account for these variables. Rather than simply measuring changes in tumor volume and counting metastatic lesions, alterations in tumor progression and the response of human immunocompetent cells need to be characterized using a variety of methodologies (Fig. 1), including gene-arrays (Figs 2,3), RT-PCR, enzyme-linked immunosorbent assay (ELISA) and fluorescence-activated cell sorter (FACS) analysis. The application of improved cellular and molecular analyses to monitor the changes within human tumor xenografts that occur following the administration of therapeutic agents will lead to a better understanding of how these agents function to limit the *in vivo* growth and metastasis of human tumors (Fig. 3). Changes in gene and protein expression within the xenograft must also be quantified and correlated with alterations in tumor growth and/or the expansion of effector cells (Fig. 1).

Future human–SCID mouse chimeric models for evaluating cancer immunotherapies will probably include a greater use of the engraftment of fresh patient tumor tissue (i.e. Model Five), to establish xenografts in the context of an intact human tumor microenvironment (Fig. 1). This is the only current model that will allow investigators to assess the role that TILs and other human stromal cells play in the response to therapy. Moreover, this model can be used to determine how closely the *in vivo* behavior of the cells within human tumor xenografts in SCID mice correlates with the clinical progression of the disease in cancer patients.

Finally, for each of the models discussed, it must be determined to what degree the responses to therapy in SCID mice reflect the responses that are observed in human patients. This can be achieved by directly comparing the data obtained from the SCID mouse xenografts with data obtained from cancer patients before and after therapy (Fig. 1).

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